REMARKS

I. INTRODUCTION

The amendment to the specification corrects an obvious typographical error. A number of the amendments to the dependent claims improve literal antecedent basis without changing meaning or scope.

A number of the claims directed to polypeptides of the invention have been amended to recite fibrous or filamentous polymers or fibers that are comprised of polypeptides of the invention. Such claims find support throughout the application as originally filed (see, e.g., specification at p. 28, line 3, to p. 31, line 19) as well as claims previously presented. (See, e.g., claims 120 and 139 pending at the time of issuance of the Office action.)

Claims 144-145 are new independent claims similar to former dependent claims 117-118, which the examiner had previously indicated would be allowable if rewritten in independent form. They are now believed to be allowable.

Claims 124-138 have been amended to specify polypeptides (or fibers comprising the polypeptides) in which specific amino acids in SEQ ID NO: 2, namely position 2 or 184, are changed. In this respect, the claims are similar to allowable claims 144-145.

II. THE FINALITY OF THE OFFICE ACTION WAS WITHDRAWN

The Applicants acknowledge with thanks the telephonic interview with Examiner Brannock February 20, 2004, in which the Examiner agreed to withdraw the "finality" of the Office action in view of the Applicant's claim amendments in the response and RCE filed October 27, 2003. For example, Applicants amended claim 87, which prompted the Examiner to respond by issuing a new rejection. If the Applicants' understanding of the interview on this issue is incorrect, then the Applicants request that this submission be treated (additionally) as a Notice of Appeal, with any necessary appeal fee charged to Deposit Account No. 13-2855.

III. REQUEST FOR INTERVIEW

The Applicants request that the Examiner contact the undersigned attorney to schedule an interview to discuss the merits of this application prior to issuance of a further office action.

IV. THE REJECTION UNDER §102(B) BASED ON GREGORI ET AL. SHOULD BE WITHDRAWN.

At pages 2-6 of the Office action, the Examiner maintained a rejection of claims 65, 67, 101, 119, 120, 139, and 141 under 35 USC §102(b), alleging Gregori et al. ("Gregori") anticipated the claims.

The Applicants continue to traverse the original rejection for all reasons previously presented. The Applicants note that the rejection (as articulated in the last action) is premised on presumptions, inferences, and interpretations of a document which, viewed in a light most favorable to the examiner's position, is silent about features of the pending claims. A proper anticipation rejection cannot be premised on presumptions and inferences relating to limitations that are not present in the cited art. *In re Robertson*, 169 F.3d 743, 745 (Fed.Cir.1999) ("Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient."); *In re Felton,* 484 F.2d 495, 500, 179 USPQ 295, 298 (CCPA 1973) ("Anticipation of inventions set forth in product claims cannot be predicated on mere conjecture respecting the characteristics of products that might result from the practice of processes disclosed in references.")

In the last paragraph of the rejection, the Examiner acknowledges that no polymer is formed: "Applicant argues that the proteasome/$A\beta^{Au}$ complex was shown not to form polymers. . . . The examiner can think of no reason why it would and nor does he understand the relevance of this to the instant rejection." The relevance to the rejection is that some of the pending claims *were directed to polymers*. More specifically, the rejected claims, as presently amended, are directed

to fiber *polymers*, and such claims cannot be anticipated by a reference that fails to disclose polymers. This is especially true where, as here, there is unrebutted testimony from a scientist in the field that steric hinderance (caused by the nanogold on Gregori's peptides) would be expected to prevent formation of higher ordered aggregates of the peptides in question. (Lindquist Rule 132 Declaration at 2.9.)

The Examiner continues to assert that column 1 of page 60 of the Gregori reference says that "the labeled peptide forms ordered aggregates." (See, e.g., Office action at p. 3.) The Applicants request that the Examiner re-evaluate this "Results and Discussion" portion of the document once more, taking careful consideration of the "Experimental Procedures" and Figure legend portion of the document as well. Specifically, the procedures used for the electrophoretic analysis of proteosome-Aβ complex formation are described at page 59, column 1, *second paragraph*. There is no mention whatsoever of the use of gold labeled amyloid beta in the *second paragraph*. In fact, gold labeled Aβ is not even introduced in the Experimental Procedures until the *third paragraph* on this page, where the term "$A\beta^{Au}$" is introduced to describe it. Not until the *fourth paragraph* of the "Experimental Procedures" does Gregori discuss using the gold-labeled amyloid beta in (a separate set of) proteasome "cross-linking" experiments.

The figure legends track the "Experimental Procedures." Specifically, the *second paragraph* procedures match with Figure 1 and with the text on page 60 whose interpretation is in dispute. There is no mention whatsoever in the Figure 1 figure legend of gold labeled Aβ or of "$A\beta^{Au}$". The data for the *third, fourth, and fifth paragraph* procedures match with Figures 2, 3, or 4. Both the procedures and the Figure legends refer to gold labeled amyloid beta and use the term "$A\beta^{Au}$".

Any remaining ambiguity is further resolved by the sentence in column 1 of page 60 that "The results in Figs. 1 and 2 indicate that Aβ, as well as gold labeled Aβ, forms complexes with proteasomes." The Figure 1 experiments were performed with Aβ, exactly as Gregori wrote, and the Figure 2 experiments with $A\beta^{Au}$.

As the foregoing discussion indicates, when the entirety of the Gregori document is reviewed for what it teaches, then the interpretation offered by the Applicants in their prior submissions (including the Lindquist declaration) is the only

reading is reasonable. Neither page 60 nor any other page of the Gregori reference teach that labeled peptide forms aggregates, and certainly not ordered polymers. (Not all protein aggregates are ordered polymer aggregates as described in the application.)

For these and other reasons, the rejection under §102(b) should be withdrawn.

V. THE REJECTIONS UNDER §103 SHOULD BE WITHDRAWN.

A. The rejection based on King and Gregori should be withdrawn.

At page 6 of the Office action, the Examiner maintained a rejection of claims 102-110, 116, 119-122, 124-126, 132, 134, 135, 137-140, 142, and 143 based on a combination of King et al. ("King") and Gregori et al., for reasons "set forth previously" and set forth with respect to the anticipation rejection based on Gregori et al. The Applicants respectfully traverse.

1. No suggestion or reason in the cited art to use a gold label to study aggregation.

Although the Examiner has, in previous Office actions, asserted that Gregori discloses that gold-labeled amyloid beta peptide formed ordered aggregates, the Examiner has now conceded that *no such aggregates were taught in the primary reference*. This acknowledgement of the true teachings in the primary reference should necessitate withdrawal of the rejection, *because there is no suggestion in the cited art that the sequence-modified, gold-labeled amyloid beta self-aggregates into higher ordered aggregates*. Instead of withdrawing the rejection, however, the Examiner has maintained the rejection based on *speculation* about situations not disclosed or suggested in the reference. Specifically, the Examiner has now alleged that, because the reference teaches that *wild type* amyloid beta rapidly aggregates at concentrations above 200 μM, "such conditions were presumably not used here." This analysis about what might happen at concentrations which "presumably" *were not tested and reported in the cited art* is both irrelevant and improper.[1] To the extent

[1] Other instances of improper speculation and misreading of the primary reference are discussed below.

Gregori suggests anything, Gregori suggests *not* to use the higher concentrations that are the subject of the Examiner's speculation. The plain facts are that Gregori neither discloses nor suggests that the sequence-modified, gold-labeled amyloid beta, or any other gold-labeled polypeptide, self-coalesces to form higher ordered aggregates. Irrespective of whether the gold-labeled peptide forms aggregates at any concentration, the plain facts are that (1) none of the cited prior art, including Gregori, teaches or suggests that the gold label is useful for studying amyloid beta aggregation; and (2) as conceded by the Examiner, Gregori used the gold labeled peptides under conditions where aggregates were not observed to form, and did not suggest that they should be used under higher concentrations, and did not suggest that aggregates of the gold labeled peptide would form under any conditions, let alone ordered polymer aggregates as recited in the claims. (See Discussion above in Section IV.)

The Examiner had originally premised the rejection on an allegation that Gregori discloses "an improved method of monitoring prion-like aggregation with electron microscopy. . . ." (See April 2003 Office action at p. 6. See also p. 7: "Gregori et al. disclose an improvement over the method used by King, e.g., Gregori discloses an improvement in the method of electron microscopy.") It is now conceded that Gregori does *not* disclose that gold-labeled amyloid beta self-aggregates, and is not a study about self-aggregation at all, but rather a study about a single amyloid beta molecule binding to the 20 S proteasome. (See title; abstract; or entire document.) In fact, it is clear from Gregori that the gold was *not* selected to study how amyloid beta aggregates with itself. Instead, the gold label was chosen to permit characterization of "the 20 S proeasome-Aβ complex" and to establish "the spatial localization of Aβ in the complex." (See p. 58, last paragraph.) As stated by Gregori, "In order to establish the spatial localization of Aβ within the proteasome-Aβ complex, we labeled an Aβ variant containing a cysteine residue as the last amino acid ($A\beta_{1\text{-}39C40}$) with 1.4nm Nanogold which could be detected by electron microscopy." (P. 60, second full paragraph.) The gold label was chosen with this *specific localization purpose* in mind, notwithstanding the concern that "we cannot exclude that ***coupling of Nanogold to Aβ may change the peptide binding affinity for the proteasome***." (P. 60, paragraph bridging first and second columns, emphasis added.) The gold labeling was chosen, despite this potential drawback, for the

specific purpose of tracking the localization of the amyloid beta peptide to (what was discovered to be) the center of the proteasome circular projection. (P. 60, last paragraph, and Figure 4.) Gregori performed an experiment to track a *single gold particle* on a single amyloid beta peptide in a proteasome. (See Figure 4 description: "Each dot represents one gold label.") A person skilled in the art understands that Gregori did not choose the gold label to monitor amyloid beta self-aggregation. A skilled person would not chose to do so, due to the risk that a gold label might "change the peptide binding affinity" and/or conformations that the peptide might adopt. (See Gregori at p. 60; Lindquist Declaration at 2.9.)

In previous actions, the Examiner also has alleged that "the motivation" to combine Gregori with King "is provided by King et al. who demonstrate the importance of monitoring prion-like aggregate formation of the Sup35 protein." As explained in the preceding paragraphs, a person reading King would have had no "motivation" to look to Gregori at all (let alone for improvements), because Gregori does not concern itself with prion *aggregation* or even with amyloid beta self-aggregation.[2] Rather, Gregori was concerned with *localizing* a single amyloid beta molecule in a proteasome complex. To the extent that Gregori would have taught anything relevant to *aggregation* to a person familiar with King, it would have been the *warning* that Nanogold may change binding affinity of the peptide to which it is bound. A person of ordinary skill would have considered this to be a needless variable to introduce, considering that King already has numerous methods for monitoring prion aggregation that do not require alteration of the protein amino acid sequence or attachment of the large nanogold particle. In other words, even if a person of ordinary skill would have combined the two references, the person would have been *dissuaded* from trying to make the claimed invention because of concerns that gold will cause anomalous behavior. The Patent Office's repeated premise that Gregori discloses an "improvement" over the method taught by King is simply incorrect, because Gregori has nothing to do with King, and

---

[2] In this regard, it bears repeating that the Examiner's initial reading of Gregori as disclosing that labeled amyloid beta forms ordered aggregates was *incorrect.*

the combination would not have been viewed as "an improvement" by persons of ordinary skill, absent hindsight knowledge of the present invention.

2. Gregori should be read for what it teaches, and not read with hindsight to try to reconstruct the present invention.

The rejection should be withdrawn for all of the reasons stated in the preceding section. This section rebuts additional allegations that allegedly support the rejection.

Gregori explicitly states that Gregori uses the term $A\beta^{Au}$ to refer to gold-labeled amyloid beta with a final cysteine residue, and uses that term throughout the manuscript. Notwithstanding, the Examiner cites *one instance* in which the Examiner believes that the term Aβ may have been used to refer to gold-labeled amyloid beta, and then concludes from this one instance that "these terms can often be used interchangeably." This represents another instance of unwarranted speculation, rather than an interpretation of the reference for what it says or would have taught one of ordinary skill at the time the present application was filed. A fairer interpretation based on the document as a whole is provided above in Section IV.

Similarly, the Examiner alleges that Gregori is "clearly referring to the fact" that additional analysis of gold-labeled amyloid beta could not be performed because this form, like the wild-type form, exhibits self-aggregating behavior. In fact, the plain language of the reference is language of *speculation*: namely, that lack of binding saturation "could be due" to the tendency of Aβ to rapidly aggregate at concentrations above 200 μM. When assessing what a reference would have taught one of ordinary skill, it is improper to read a statement of speculation – *clearly not tested by the authors* – as "clear fact." The fact that Aβ peptides self aggregate at high concentration does not meant that $A\beta^{Au}$ would self-aggregate under high concentrations. The Examiner instead should look at the fact that the references teaches away from using high concentrations, and the Lindquist declaration that explains that nanogold would reasonably have been expected to alter aggregation properties.

The Examiner places repeated emphasis on one sentence, repeated below in bold, which, when read in context, fails to support the Examiner's position:

> The results in Figs. 1 and 2 indicate that Aβ, as well as gold-labeled Aβ, forms complexes with proteasomes. Furthermore, $A\beta^{Au}$ was able to inhibit ubiquitin-dependent degradation of lysozyme with only a slight increase of the IC_{50} when compared with $A\beta_{1-40}$ (Fig. 3). **As already discussed, no further kinetic analysis could be performed due to the anomalous behavior of Aβ in solution.** Our result indicates that $A\beta^{Au}$, similar to unlabeled Aβ, interacted with the 26 S proteasome . . . .

It is clear from the surrounding sentences and cited figure that the authours are using the term "Aβ" to refer to the *wildtype* amyloid beta (without cysteine modification or gold particle) and use terms such as "$A\beta^{Au}$" or "gold-labeled Aβ" when they wish to refer to gold labeled amyloid beta. (See Discussion in Part IV, above.)

3. The Examiner accorded insufficient weight to an expert affidavit

The inventor, Dr. Lindquist, has experience working with nanogold and self-aggregating proteins and has provided her opinion that, "Based on my experience with Nanogold and with aggregating proteins such as prions, I would not predict, and *do not believe that a person of ordinary skill in the art would predict*, that Gregori's cysteine-substituted, Nanogold-labeled Aβ would self-aggregate into higher ordered aggregates as contemplated in the patent application. Instead, the Nanogold would be expected to cause steric hinderance to inhibit aggregation." The Examiner

appears to have dismissed this experienced opinion in favor of his own opinion, without any sworn statement or other basis for doing so.[3]

Finally, the Examiner refuses to accord weight to the declaration because of one clause ("aggregates as contemplated in the patent application") that clearly is referring to SCHAG aggregates. Any doubt should have been erased by the very next sentence, which states, "Instead, the Nanogold would be expected to cause steric hinderance to inhibit aggregation."

Gregori provides no evidence of suggestion that Gregori's gold-labeled amyloid beta beta is aggregating at all, let alone aggregating to form polymers/fibers. In contrast, at least one recent publication has reported that amyloid beta peptides (Aβ40) attached to colloidal gold particles at the C-terminus of the peptides, via a thioester bond, do *not* form fibrils. (See Kayed et al., *Science* 300: 486 (2003) (Exhibit A hereto, especially supporting on line materials at p. 8: "They are very stable, homogeneous and without contamination from either low MW Aβ or fibrils (Fig. S1B).") The Gold particles used by Kayed et al. were of a mean diameter of 5.3 nm (p. S1) and, apparently, attached to multiple Aβ peptides (Fig. S1A) to mimic the structural organization of Aβ oligomers. Assuming that the Kayed et al. model for oligomer formation is correct, and that the C-terminus of Aβ peptides must coalesce

---

[3] The Examiner places much emphasis on a statement that a Nanogold particle is 1.4 nm in diameter, comparing that length to an alleged "five amino acid" polypeptide shown in a Alberts, a molecular biology textbook. In actuality, the peptide shown in Alberts is *fifteen amino acids* in a beta sheet conformation. Moreover, these fifteen amino acids are shown to be about 1.39 nm in length at their *longest dimension*, with other dimensions apparently shorter, which suggests that the fifteen amino acid beta sheet may be smaller than the nanogold in other dimensions. More importantly, Gregori provides direct evidence -- in the form of SDS-PAGE data -- that the nanogold is *behaving* like a moiety of perhaps 70% or more of the total size of the Aβ, as stated in the Lindquist declaration. The Examiner "does not understand how the behavior of a gold particle in an SDS-PAGE gel could provide any information as to the real molecular weight or the size of the gold particle." (Office action at p. 5.) The SDS-PAGE procedure separates molecules by size, shape and charge in a detergent environment, and therefore provides indirect information about the size of the gold particle, and more particularly, direct information about the way that it affects the *behavior* (migration) of amyloid beta due to affects on size, shape, and charge. These properties (size, shape, change) all affect protein conformation and binding properties.

together in a structure shielded from the aqueous environment, it is not hard to understand why Gregori's Aβ particles -- each attached to a gold particle at the C-terminus, would suffer from steric hindrance that prevent aggregation.

For all of these reasons, it was improper to dismiss the Lindquist declaration and its evidence relevant to the issue of unobviousness. In the absence of experimental data, the Examiner is compelled to consider the statements presented in the Declaration, which was executed by a scientist with years of experience in the field, as scientifically accurate and a fair statement of how one of ordinary skill in the art would have interpreted the teachings in the art.

B. The rejection based on Prusiner (US 5,750,361) and Stayton should be withdrawn.

At pages 6-11 of the Office action, the Examiner maintained a rejection of claims 65, 67, 81, 101, 119, 120, and 139-141 under §103 based on a combination of U.S. Patent No.: 5,750,361 ("the Prusiner patent") and Stayton et al. The Applicants respectfully traverse.

1. The rejection is moot with respect to claims 120 and 139-141.

At the outset, claim 120 has been canceled by the instant amendment and claim 139 (and thus dependent claims 140 and 141 therefrom) amended to depend from new claims 144-145. Claims 144-145 are independent claims similar to former dependent claims 117-118, which the examiner had previously indicated would be allowable if rewritten in independent form. In view of these amendments, the basis for rejection of claims 120 and 139-141 is believed to be moot.

However, the Applicants continue to traverse the basis for the rejection for all reasons previously stated. The rejection as articulated in the most recent action is addressed in the next section.

2. The Examiner has failed to establish a prima facie case of obviousness.

Section 706.02(j) of the MPEP summarizes three basic criteria to establish a prima facie case of obviousness based on two or more references. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all of the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure. These criteria have not been satisfied.

a. The scope and content of the prior art.

In characterizing the primary reference, the Prusiner patent, the Examiner alleged that "U.S. Patent No: 5750361 discloses method of assaying formation of prion complexes (i.e. SCHAG amino acid sequences) by constructing polypeptides comprising prion aggregation domains labeled using materials and methods well known in the art including florescent dye and spectrophotometrically-detectable chromophores (see col 11 brdiging co 12). U.S. Patent No: 5750361 discloses assays to determine that the labeling occurs at position exposed to the environment, i.e. that the label does not interfere with complex formation (e.g. col. 11 and 12)."

This characterization of the Prusiner patent's teachings is both incorrect and based on a faulty assumption. It is a faulty assumption to equate a determination that a label is in a position exposed to the environment in an ordered aggregate with a determination that a label does not interfere with complex formation. In actuality, some labels, such as radioisotopes, are sufficiently small and/or replace atoms in amino acids in a manner that they do not materially affect the 3-D structure, charge, or other properties of the peptide and would not be expected to interfere with complex formation, irrespective of whether they are "exposed to the environment" or buried within the three dimensional structure of a peptide, or buried within the three

dimensional structure of a peptide aggregate. Moreover, depending on the size and folding of proteins, a label may be distant from surfaces or domains involved in complex formation (and not interfere with complex formation), yet still be folded into a pocket of the protein that is not exposed to the environment. (In other words, a protein may contain regions that are neither exposed to the environment nor involved in self-aggregation.) Furthermore, a label theoretically could be attached to a peptide at a position that would be exposed to the environment in an aggregate, but nonetheless prevent the peptide to which it is attached from ever adopting a conformation that can form ordered aggregates.[4]

The Patent Office's characterization of the Prusiner patent also is inaccurate in that Prusiner never teaches to evaluate whether a label interferes with complex formation. In fact, as discussed in the following paragraphs, the Prusiner patent does not even teach that one should try to detect or quantify a label in a PrP complex.

Referring to column 11 of the Prusiner patent, Prusiner teaches a first sedimentation assay in which a test compound is contacted with PrP^c, a PrP peptide is added to the test compound/PrP^c mixture, and the formation of a prion protein complex is detected using procedures that involve measuring the presence and/or quantity of insoluble complex formed, such as sedimentation, protease resistance, and conformation. (Col. 11, lines 8-25.) Prusiner does not teach or suggest to use a label in this assays or teach or suggest that there is any value in trying to modify a PrP protein to attach a label or try to form a complex with a detectable label for this assay.

In a second assay (col. 11, lines 45-56), PrP^c is "derivatized, e.g., with Streptavidin," *for the purpose of attachment of the PrP to a solid support such as an*

---

[4] In this regard, it is important to recognize that the present application teaches that SCHAG polymer aggregates can be formed, and attachments of interest can be attached to a reactive side chain that is exposed to the environment in the ordered aggregate *after* the aggregate is formed. This approach of attaching interesting functionalities to aggregates after aggregate formation is neither disclosed nor suggested in the Prusiner patent, the Stayton article, or any other art of record.

assay plate.[5] This embodiment in column 11 involves binding an anti-PrP monoclonal antibody to the plated-bound PrP^c, and screening for compounds that can displace the antibody. Binding of a test compound is detected by *determining the amount of free antibody*. Again, there is no teaching or suggestion to label PrP at all for this assay. The detection involves measurement of *free antibody*.

In a third assay (bridging cols.11-12, cited by the Examiner), a labeled PrP peptide is used to form a complex with PrP^c, and *the displacement* of labeled PrP peptide (by PrP^{Sc}) from the complex is measured to assay for the presence of PrP^{Sc}. Either the PrP peptide or the PrP^c may be labeled, but importantly, "the displaced peptide molecules are *measured in the supernatant fraction* after ultracentrifugation [to remove the insoluble PrP^c/PrP^{Sc} complexes that may have formed]." As explicitly stated in the Prusiner patent, "the amount of displaced labeled peptides is measured in the unbound phase." (Col. 12, lines 15-26.) An advantage of this assay, according to the Prusiner patent, is that PrP^{Sc} does not need to be soluble and PrP^c does not need to be removed prior to detection. The assay circumvents a "PrP^{Sc} solubility problem." (Col. 12, lines 40-48.) There is no teaching or suggestion to attempt to detect or measure label in a prion fiber. There is no teaching or suggestion that a label should be attached at a position that is exposed to the environment in a prion fiber. This concern is simply not stated to be, or recognized to be, relevant to the Prusiner assay embodiments. In the Prusiner patent, "The presence of PrP^{Sc} in a sample is determined from the *displacement* of a PrP peptide from the prion protein complex."

b. No motivation to combine the Prusiner patent and Stayton et al

A brief review of the cited references helps elucidate that the obviousness rejection was improper. The Prusiner patent is concerned with finding cures to prion diseases, and purports to teach assays for identifying compounds that

---

[5] See also col. 12, lines 27-39, an excerpt cited by the Examiner at page 7 of the Office action. Here too, the biotin attachment is for the stated purpose of attachment to a streptavidin coated plate, and not for purposes of detection as suggested by the Examiner.

inhibit the binding of PrP peptides to cellular PrP (PrP^C) and assays to measure the pathogenic scrapie form of PrP (PrP^{Sc}). The assays that Prusiner teaches are discussed above.

The Prusiner patent also explains that the PrP peptides have significant alpha helix structure and are "substantially devoid of β-sheet conformation," whereas PrP^{Sc} has "high β-sheet content." (See, e.g., Col. 4, lines 3-19; col. 6, line 64, to col. 7, line 21; col. 7, lines 42-50; and col. 8, lines 11-25.)

The Stayton reference has nothing whatsoever to do with proteins that self-aggregate into higher-ordered aggregates, or finding compounds to inhibit aggregation, or measuring pathogenic aggregrates of such peptides. The Stayton reference has nothing whatsoever to do with a displacement assay to measure the formation of a prion particle. The Stayton reference involved an attempt to "quantitate binding interactions" between cytochrome b_5 with either of two other proteins (cytochrome P-450 or metmyoglobin) with which it forms complexes in redox electron transfer pathways. (See introduction at p. 13544.) Elegant prior work of others had already established structural models of the proteins involved. Stayton characterizes its own work as a "new approach" to defining complex formation in which a single cysteine was introduced into the cytochrome b_5 sequence, to which an "environmentally sensitive" fluorescent probe was attached. Three dimensional structures of the involved proteins established by prior art studies, structural NMR, and x-ray crystallography were necessary to perform the analysis conducted by Stayton.

A threshold issue for obviousness based on two or more references is whether there is a legally recognized motivation to combine the references. At page 8 of the Office action, the Examiner asserts that the motivation to combine the Prusiner patent and Stayton et al "...was provided by U.S. Patent No: 5750361 wherein it is stated that the polypeptide "should be modified as described in the art" and that the amino acids could be substituted as long as the change does not effect complex formation (col 7, L30-36) and by Stayton et al who provide methods of labeling a polypeptide, wherein the labeled polypeptide is useful for detection of complex formation."

The Examiner's reasoning fails to withstand scrutiny. First, the patent does not say "should" but instead says "may." The distinction is subtle but significant, in the context of whether the Patent provides a *motivation* to alter the invention.

Second, the two references relate to disparate fields of study, one concerned with finding inhibitors of the self-aggregation of prion protein into pathogenic, highly resistant, stable, insoluble particles, and the other concerned with further elucidation of the well studied complex interactions between cytochrome proteins involved in electron transfer biochemical pathways. No reason is given as to why a person involved in the study of pathogenic self-aggregating proteins would turn to literature involving cytochrome redox protein-protein electrostatic interactions. The Examiner asserts that "the steps of choosing an amino acid residue in the sequence having a side chain that is exposed to the environment and substituting this amino acid with one have a reactive side chain" are "old and well established [in] the art of protein complex detection." However, this allegation is belied by the Examiner's failure to cite any such reference that, for example, is concerned with self-aggregating proteins, i.e., a subject matter of the present application or of the primary reference. In fact, Stayton is not concerned with complex detection per se, but rather, with quantitating binding interactions and elucidating the binding orientation of different molecules in the cytochrome redox protein pathways.

Moreover, as explained in the prior response (including the Lindquist Declaration), one of ordinary skill in the field of the invention (or in the field of the Prusiner patent) would have been *dissuaded from attempting to combine* Prusiner's teachings with Stayton's because of the unique properties of prion proteins. As explicitly discussed in the Prusiner patent, the prion protein has a *different conformation* in its soluble state versus its PrP^{Sc} aggregation state. At the time of the invention, the structural data that Stayton used to select cytochrome b_5 residues for modification was neither available nor obtainable for SCHAG proteins. (Lindquist Declaration at 3.4 to 3.5.) Even if available, it would have been considered to be of limited value because the structure of SCHAG proteins in a theoretical crystalline

state would not necessarily represent the structure in the soluble state or the prion (aggregate) state.

In this regard, it is important to emphasize that the Examiner has failed to identify any deficiency in the Prusiner patent that would have motivated a person to look outside that patent in the first place, let alone look outside the patent and try to adopt an approach with the inherent incompatibility of Stayton. The excerpt from the Prusiner patent cited by the Examiner states, "The PrP peptide may have modifications of the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 10, e.g., one or more amino acid changes, one or more amino acid deletions, and/or one or more amino acid insertions, so long as it retains the characteristics of having at least one α-helical domain and/or a random coil conformation in an aqueous solution, and binding PrP^C to form a prion protein complex." This excerpt does not teach or suggest that one should choose an amino acid exposed to the environment and substitute the amino acid with one having a reactive side chain. This excerpt does not teach or suggest that the peptide should be modified for the purpose of attaching a label or for the purpose of monitoring complex formation. In fact, Prusiner does not provide a relevant motivation for making the modifications. The Stayton reference does not complement Prusiner in this regard because it provides no guidance about retaining alpha helical domains or random coil conformation of prion proteins in aqueous solution.

Upon reading the above excerpt from the Prusiner patent, one of ordinary skill in the art would not look to Stayton et al for a procedure to substitute a surface-exposed amino acid with an amino acid with a reactive side chain and to then further modify the polypeptide by labeling the reactive side chain with a substituent conducive to the study of protein-protein interactions. Even if there were a motivation to look "in the art" for guidance of how to modify Prusiner's PrP protein primary sequence, one would not have been motivated to look to Stayton.

The Applicants raised the incompatibility of Prusiner and Stayton in their prior submission, a point which appears to be unrebutted and unrebuttable. Instead of withdrawing the rejection, the Examiner alleges that "Stayton is being relied upon only to demonstate the concept, that is widely appreciated in the art, that

if one whishes to study protein/protein interactions using the various available labeling techniques, then one would be motivated to [modify] residues that are exposed to the environment when the protein complexes are formed, and that this can be accomplished by replacing that residue with one having a reactable side chain and then further modifying the side chain with a fluorescent dye. This is a very simple concept obvious to anyone skilled in the art."

The inconsistency of the Examiner's opinion is clear: it is improper to say, on the one hand, that Stayton provides part of the motivation to combine Stayton with Prusiner and then, when the references are shown to be incompatible, to say that Stayton is being relied upon only to demonstrate a "simple concept obvious to anyone skilled in the art." The Federal Circuit has repeatedly warned that an examiner cannot substitute an intuitive feeling about what should be simple and obvious to anyone in the art for actual teachings of references for which there is a true motivation to combine.[6]

The Examiner continued by stating that there are "two simple reasons" why "this is a very simple concept . . . obvious to anyone skilled in the art." First, the Examiner alleges "that modification of residues buried in the interior of the complex would be more likely to affect the conformational stability of the complex, and" second, "if the reporter conjugate is buried in the interior of the complex, then the signal emanating from that conjugate is likely to be diminished." In answer, the

---

[6] See *In re Rouffet*, 149 F.3d 1350, 1357 (Fed. Cir. 1998) ("To prevent the use of hindsight based on the invention to defeat patentability of the invention, this court requires the examiner to show a motivation to combine the references that create the case of obviousness."); *In re Sang Su Lee*, 277 F.3d 1338, 1344-45 (Fed. Cir. 2002) ("This factual question of motivation is material to patentability, and could not be resolved on subjective belief and unknown authority. . . . . "Thus when they rely on what they assert to be general knowledge to negate patentability, that knowledge must be articulated and placed on the record. The failure to do so is not consistent with either effective administrative procedure or effective judicial review. The board cannot rely on conclusory statements when dealing with particular combinations of prior art and specific claims, but must set forth the rationale on which it relies."); see also *In re Kotzab*, 217 F.3d 1365, 1369-70, 55 USPQ2d 1313, 1316 (Fed. Cir. 2000) (to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of *the desirability of making the specific combination* that was made by the applicant.)

Applicants again point out that the Prusiner patent does NOT teach to be concerned with whether or not a label is buried within a PrP complex, and does NOT teach (e.g., at column 7) that the amino acid sequence should be modified for the purpose of attaching a label. Instead, Prusiner teaches to detect a label on a polypeptide that has been *displaced from a complex*, i.e., to detect a label measured in the unbound phase (column 12).

Moreover, Stayton teaches exactly the OPPOSITE of what the Examiner has reasoned. Specifically, Stayton teaches to use a label that is "environmentally sensitive" because Stayton is not concerned with producing a complex in which a strongly detectable label is present on the surface. Rather, Stayton is concerned with identifying how two proteins interact, and Stayton is provided with valuable information for Stayton's purpose when the fluorescence emissions of Stayton's label are altered due to protein-protein interaction.

At the bottom of page 9, the Examiner explains that he did not understand the Applicant's "no motivation to combine" evidence at paragraph 3.8 (Lindquist) and at page 13 of the prior submission. The Applicants' point was that the law forbids using the present application and claims as a hindsight roadmap to build the invention from disparate teachings in the prior art. If one reads the Prusiner patent in the context of what Prusiner was trying to achieve, rather than the context of what the Applicants describe as their invention, then the logic behind the rejection falls apart. Prusiner was interested in methods for screening for compounds that inhibit prion formation, and Prusiner teaches perfectly adequate techniques involving, e.g., sedimentation or protease resistance. It would be illogical to try to modify Prusiner by looking to references that involve expensive and time-consuming techniques (e.g., structural NMR or x-ray crystallography), especially when the reference is from a totally unrelated field and provides no expectation of an improvement to Prusiner's own techniques.

c. Failure to address all elements of the claimed invention

Some of the pending claims are directed to polymers of the invention attached to a solid support. (See, e.g., claims 138, 140.) The Examiner has suggested that this limitation is met, e.g., by column 11, lines 48-50, of the Prusiner patent. In actuality, this excerpt from the Prusiner patent is concerned with attaching PrP^c to a solid support, in an assay to determine whether candidate compounds can displace an antibody bound to the PrP^c. The excerpt does NOT teach or suggest attaching a polymer of the present invention to a solid support.

The rejection alleges that it would have been obvious to produce *a polypeptide* comprising a prion aggregation domain, for use in an aggregation assay, and thus producing the aggregates themselves, labeled with a fluorescent or other spectrophotmetrically-detectable substituent . . . . As explained in detail above, the Prusiner patent does not teach to make isolated aggregates with labeled peptides. Rather, the Prusiner patent teaches to use labeled peptides in displacement assays, where (unlabeled) PrP^{Sc} aggregates are detected by their ability to displace labeled peptide from a complex bound to a plate. This difference cannot be ignored with respect to the rejected claims that are presently directed to polymers, and not to peptides.

The present claims recite, e.g., substitution of an amino acid in a SCHAG polypeptide with an amino acid having a reactive side chain that will be exposed to the environment in a polymer comprised of the polypeptide. The Examiner alleges that the Prusiner patent alleges that the polypeptide "should" be modified (including substituted) "as long as the change does not [affect] complex formation." As explained above in detail, selection of amino acids to substitute so as to preserve complex formation is NOT equivalent to selection of amino acids that have (reactive) side chains that will be exposed to the environment in an ordered polymer peptide aggregate. The Stayton reference does not cure this defect for reasons noted above. Stayton teaches nothing whatsoever about identifying the position of amino acid side chains in ordered polymer aggregates of a SCHAG polypeptide.

The Examiner alleges (p. 8) that Stayton is being relied upon "only to demonstrate the concept, that is widely appreciated in the art, that if one wishes to study protein/protein interactions using the various available labeling techniques, then one would be motivated to [modify] residues that are exposed to the environment when the protein complexes are formed . . . . In actuality, Stayton described selecting amino acids of cytochrome b_5 that are allegedly exposed to the environment of an isolated cytochrome b_5 peptide. Stayton's process of identifying an amino acid that is exposed to the environment *of a polypeptide monomer* (e.g., Stayton's cytochrome b_5) is distinct from the process of identifying an amino acid of a polypeptide monomer that will be exposed to the environment in a highly ordered polymer aggregate formed from multiple copies of the polypeptide monomer. The Stayton article does not pertain to such polypeptides and provides no teachings or suggestions whatsoever that are relevant to this limitation of the claimed invention. Neither the Prusiner patent, nor the Stayton reference, nor any other document of record teaches or suggests to identify and modify an amino acid of a polypeptide monomer that will be exposed to the environment of an ordered polymer aggregate formed from multiple copies of the monomer.

The Examiner alleges (p. 9) that "methods for determining what residues are exposed to the environment are well known in the art, as is essentially admitted in the instant specification . . . . This too, is legal error, as it is inappropriate to cite to the Applicants' enabling disclosure in the application as evidence of what was allegedly obvious. What is obvious must be judged from the prior art as a whole, not the applicant's specification.

The Examiner further alleges (p. 9) that the Prusiner patent "discloses assays to determine that the labeling occurs at positions exposed to the environment, i.e., that the label does not interfere with complex formation (e.g., col 11 and 12)." (See also Office action at p. 10: "one simply determines that the labeled residues do not inhbit comples formation as taught in the Prusiner patent, e.g. col 11 and 12.") Clarification is requested. The Applicants discuss the assays at column 11-12 of Prusiner in detail, above. None of the assays involve detecting a label in a complex. Nothing in these columns teaches to use any assay to determine whether a label

occurs at a position exposed to the environment of the peptide alone, or the peptide in a complex. Labels are used in the Prusiner patent to detect displaced peptide in a soluble state. Assays such as sedimentation, protease resistance, and conformation are used to detect formation of insoluble complexes (col. 11, lines 13-20), and these assays do not involve a label as suggested by the Examiner. Even if (for the sake of argument) Prusiner discloses assays that *could be used* to determine if a label is exposed to the environment, Prusiner does not teach to make this determination. Even if Prusiner teaches that amino acid substitutions can be made, Prusiner does not teach that substitutions should be made for the purpose of attaching a label (or anything else) to a non-naturally occurring reactive amino acid side chain.

d. No reasonable expectation of success based on the teachings in the prior art.

As explained in the preceding section, Prusiner contains no significant guidance with respect to placement of a label; Prusiner contains no teachings regarding the making of an amino acid substitution for the purpose of attaching a label; and Prusiner only teaches detecting labeled PrP in a soluble state, displaced from a complex. Stayton, on the other hand, contains no teachings whatsoever about self-aggregating proteins. Stayton also contains no suggestion to modify a self-aggregating protein for the purpose of attaching a label that would be exposed to the environment of a polymer aggregate formed from multiple copies of the self-aggregating protein. In fact, no reference has been cited that suggests the desirability of making such an invention. In the unpredictable biotechnology arts, there can be no reasonable expectation that applying Stayton's teachings to Prusiner would be successful, and the Examiner has failed to articulate why the prior art indicates it should be successful. In fact, the Examiner has stated on the record that the Examiner does not believe that it is possible (even with the teachings of the present application) to make useful predictions about amino acid positions or identity of modified sequences that will work as required by the claims. (See rejection under §112, first paragraph.) The Examiner's subjective feelings about what would have been obvious for anyone in the art *to try* are not relevant to whether there was a reasonable

expectation of success. The Examiner's statement that it is not possible to make useful predictions (office action, p. 12) indicates that the Examiner does not believe that there is a reasonable expectation of success if one were to have modified the teachings of the Prusiner patent in a manner suggested by Stayton.

e. Conclusion

The Patent Office has failed to identify an acceptable motivation (and has instead relied upon improper hindsight) to combine the two references cited as a basis for the rejection. Even if the references were combined, they do not teach all of the limitations of the claims. Even if the claim limitations can be constructed from picking and choosing elements from the disparate prior art teachings, there is no reasonable basis for expecting that the recombination of prior art teachings would be successful as taught by the Applicants. For all of these reasons, the rejection based on the Prusiner patent and Stayton should be withdrawn.

C. The rejection based on Prusiner (US 5,750,361), Stayton, and King, and the rejection based on Prusiner (US 5,750,361), Stayton, and King, and Paushkin should be withdrawn.

The third and fourth references cited by the Examiner (to combine with the Prusiner Patent and Stayton) do not remedy any of the defects described above with respect to the primary and secondary references. (The Examiner provides no new discussion of these references in the latest action.) For these and other reasons, including the reasons set forth in the preceding amendments, the rejections should be withdrawn.

VI. THE REJECTIONS FOR LACK OF WRITTEN DESCRIPTION UNDER §112, FIRST PARAGRAPH, SHOULD BE WITDRAWN.

At pages 11-14, the Patent Office rejected claims 124, 127-131, and 134-137, and separately rejected claim 81, alleging lack of written description. The Applicants respectfully traverse.

A. The present invention meets the Patent Office's guidelines for written description of a functional protein invention.

With respect to claims 124, 127-131, and 134-137, the Examiner alleged that the claims "require an essentially limitless number of polypeptides having only 90% identity with SEQ ID NO: 2. (Office action at p. 12.) The Examiner *admits* that "one of skill in the art would reasonably predict that many of these proteins would work as required by the claims." (*Id.*) Nonetheless, the Examiner maintained the rejection, alleging that one "would not be able to make useful predictions as to the amino acid positions or identities of those sequences based on the information disclosed in the specification."

Contrary to what the Examiner has suggested, the written description provision of the statute does NOT require the ability to make predictions. Example 14 of the Patent Office's own written description training materials relates to a "percent identity" claim and a hypothetical disclosure with no stated ability to make prediction and not a single example of a working variant sequence:

> The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

On this hypothetical set of facts, the PTO training materials instruct that, "The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus." The PTO accepted in this example that the claim at issue was open-ended, and would embrace variants that were larger than the recited sequence.

The often cited case of *In re Wands* (from the Patent Office's reviewing court) also refutes the examiner's position that predictability of amino acid changes is necessary for written description. In that case, the technology involved monoclonal antibodies, and the applicants in that case did not teach how to predict working embodiments based on amino acid sequence. Rather, working embodiments were identified by *screening*.

Moreover, the Examiner is incorrect in asserting that the number of polypeptides is limitless. The present claims recite at least 90% identity, meaning that about 9 of every ten amino acids of a SCHAG sequence in SEQ ID NO: 2 are retained in a claimed sequence. This is generally regarded as "not substantial variation." (Compare PTO Example 14, discussed above.)

The Examiner likewise is incorrect in asserting that one is unable to make structure-function predictions based on the teachings in the application. Example 9, part B of the specification teaches techniques for making point mutants and screening them for properties relevant to the claims. In addition, Example 3 of the Application teaches that more substantial mutants can be constructed while preserving fiber-forming properties. In particular, the Applicants showed that the fiber forming properties of Sup35 (SEQ ID NO: 2) could be manipulated in a predictable manner by increasing or decreasing the number of PQGGYQQYN oligopeptide repeats that occur in the peptide. (See pp. 54-58.) The application also provides guidance that the percentage of polar residues (particularly asparagines and glutamine) is important for prion fiber forming properties. (See generally pp. 8-9 and Example 5.) The Application contains guidance that conservative substitutions are less likely to alter SCHAG properties. (See, e.g., p. 8.) The guidance for making modifications and the actual data in the examples place the present application in a far more favorable position for claiming variants than the hypothetical Example 14 in the Written Description Training Materials, or the cases cited by the Examiner.

The Examiner offers one final, confusing statement/misquotation for distinguishing Example 14, namely, that "In Example 14, 'procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art.'"

The Examiner has misread the example, which actually reads, "The procedures for making variants of SEQ ID NO: 3 are conventional in the art **and an assay is described which will identify other proteins having the claimed catalytic activity**." Thus, the premise of the hypothetical is that procedures for making variants of *any amino acid sequence are conventional.* This is the only logical interpretation, since the training materials clearly state the additional premise that "A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious." If SEQ ID NO: 3 is novel and unobvious, then nothing in the art could be conventional with respect to varying this sequence specifically. Rather, the logical reading of the training example is that the Patent Office has recognized that recombinant technology has matured enough to permit making variants of any sequence.

The second part of the training example says that *an assay is described* for identifying other proteins with the desired activity. The present applicants do better than this, because not only do they describe aggregation assays, they provide numerous working examples of using the assay to identify suitable variant sequences.

Consistent with Example 14 of the written description training materials, the present application describes assays for the identification of novel prion-like amyloidogenic sequences and experiments for screening the sequences to confirm prion-forming properties. For example, Example 5 of the present application describes procedures that involve screening protein sequence databases and probing polynucleotide libraries to identify sequences suspected of having prion-forming capabilities. Moreover, Example 5 further teaches that sequences identified using the aforementioned techniques may be screened to determine whether the sequences represent/encode proteins having the ability to aggregate in a prion-like manner. Example 5 describes an aggregation assay using fusion proteins and an *in vitro* aggregation assay using chaperone protein. Example 8 provides a high throughput genetic screen for identifying peptides with prion fiber-forming properties.

B. SCHAG Peptides are amenable to sequence variation.

Even though the present application meets the Patent Office's stringent structure-function criteria for adequate written description of a protein invention, it is not clear that these stringent criteria should even be applied rigorously to the present invention. The present application contains repeated guidance that SCHAG peptides preferably are rich in polar, uncharged residues (especially asparagines (N) and glutamine (Q), but also serine and tyrosine) and relatively low amounts of secondary structure in their soluble state, whereas the peptides can also adopt a beta sheet structure in connection with a polymerized state. (See, e.g., specification at p. 8, lines 29-31; p. 4, lines 7-9; p. 51, lines 12-14; p. 61, lines 8-14.) For example, whereas an average globular protein may have a Q + N amino acid content of about 8%, prion proteins such as those described in the application typically have a Q + N content in excess of 30%. [See Exhibit B, providing amino acid content of Sup35 and Ure2, broken down by domains. The N region of each molecule is the polar-residue rich SCHAG/prion domain.] At the same time, a typical globular protein will have approximately 26% charged residues (K,R,E,D), whereas in the typical prion domain, the K+R+E+D content is less than about 12%. However, while meeting these general guidelines as to amino acid content, the examples in the application, and numerous examples in nature, demonstrate that peptides with these properties are quite permissive of primary sequence variation, while still maintaining SCHAG properties. Attached as Exhibit C is an alignment of the amino acid sequences of three yeast/fungal Sup35 proteins whose sequences are specifically recited in the application. In the Sup35 N region (i.e., the SCHAG/prion domain), sequence similarity between these proteins is comparatively low: (S. cerevisiae: C. albicans ~ 26%; S. cerevisiae: P.pinus ~ 31%; S. cerevisiae: P.pinus ~ 45%), whereas similarity in the C region, responsible for protein function in the unaggregated protein, remains significantly higher. (See Exhibit B). Attached as Exhibit D is a schematic that graphically depicts these amino acid enrichment patterns in eight different species of Sup35. Whereas sequences are diverse from an amino acid alignment standpoint, i.e., comparing primary sequence, the character of the sequences are similar. Thus, the present invention - which relates to the ability of peptides to form useful fibers - does

not raise the typical primary-structure-function issues raised by many other biotechnology patent applications.

For all of these reasons, the rejection for lack of written description should be withdrawn.

VII. THE REJECTION OF CLAIM 81 IS MOOT.

Claim 81 was amended to recite a polymer, rather than a peptide, and the term "exactly" was removed, rendering the rejection of claim 81 moot.

VIII. PETITION FOR EXTENSION OF TIME AND DEPOSIT ACCOUNT AUTHORIZATION

This amendment is timely filed with a petition and fee for a three month extension of time to extend the period for response until July 13, 2004. The Patent Office is authorized to charge any other necessary fees associated with this submission to Deposit Account No. 13-2855. Reconsideration and withdrawal of all rejections is requested.

Respectfully submitted,

MARSHALL, GERSTEIN, & BORUN LLP
6300 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6357
(312) 474-6300

By: 

David A. Gass
Reg. No. 38,153

July 13, 2004

Supporting Online Materials

Materials and Methods

Peptide synthesis: All Aß peptides, prion 106-126 and IAPP were synthesized by fluoren-9-ylmethoxy carbonyl chemistry using a continuous flow semiautomatic instrument as described previously (1). The detailed synthesis of Aß C-terminal thioester analogues will be published elsewhere (R.K., S.S.M. and C.G.G). Briefly the synthesis was carried out according to the methods described (2). The first amino acid was manually coupled to the *sulfamylbutyry- AM-PEGA* resin (Novabiochem, San Diego, CA) then the peptide sequence was assembled as described above. The purity was checked by analytical RP-HPLC and electrospray mass spectrometry and determined to be approximately 95%. Human insulin and lysozyme were from Sigma (St. Louis, MO). Polyglutamine KKQ40KK was a gift from Dr. Ronald Wetzel, and α-synuclein was a gift from Dr. Ralf Langen.

Preparation and characterization of the antigen: Gold colloids (mean diameter 5.3nm) were obtained from Ted Pella Inc, The nanoparticles were incubated in a solution of 0.2 mg/ml of the C-terminal thioester Aß40, pH (5.0-5.5) for 3 hr, then the pH was adjusted to 7.4 with 100 mM Tris pH 8.0 (0.2% sodium azide). After incubation for 6 hr at room temperature, the antigen was collected by centrifugation at 30,000 x G at 4 °C for 30 min, washed three times with PBS pH 7.6 to remove unincorporated Aß and then re-dispersed in DD H_2O (0.02% sodium azide). The resulting micelle molecular mimics were analysed by AFM, circular dichroism spectroscopy, thioflavin T fluorescence (3), bis-ANS fluorescence (4), and UV/visible spectroscopy and stored at 4°C. The molecular mimics and soluble Aß oligomers have very similar structural properties on the basis of these criteria (data not shown).

Antibody Production: New Zealand white rabbits were immunized with the antigen (1ml, 0.08-0.1 mg of Aß per rabbit, dialyzed against PBS at 4 °C, overnight). For the first immunization, equal parts antigen and complete Freund's adjuvant were used, while for the subsequent 11 injections the antigen was mixed with incomplete Freund's adjuvant and injected at 2-week intervals. Animals were injected subcutaneously in small increments of 0.1 ml per site in a checkerboard fashion on the scapular region.

Preparation of Aβ intermediates and fibrils: Fibrils were prepared under three different conditions, water (pH 3.8-4.2), 10mM Tris (pH 7.4), and 50mM Tris 100 mM NaCl (pH 7.4), each containing 0.02% sodium azide. The final peptide concentration was 0.3-0.5 mg/ml (80-125 μM). The samples were stirred with a Teflon coated micro stir bar at 500 rpm at room temperature for 6 to 9 days. Fibril formation was monitored by thioflavin T fluorescence and UV light scattering. Once fibril formation was complete, the solutions were centrifuged at 14,000 x G for 20 min, the fibril pellet was washed 3x with the doubly distilled water, and then resuspended in the desired buffer. The morphology was verified by AFM or negative stain EM. Soluble oligomers were prepared by dissolving 1.0 mg Aβ in 400µL HFIP for 10-20 min at room temperature. 100 µL of the resulting seedless Aß solution was added to 900 µL DD H_2O in a siliconized Eppendorf tube. After 10-20 min incubation at room temperature, the samples were centrifuged for 15 min. at 14,000 x G and the supernatant fraction (pH 2.8–3.5) was transferred to a new siliconized tube and subjected to a gentle stream of N_2 for 5-10 min to evaporate the HFIP. The samples were then stirred at 500 RPM using a Teflon coated micro stir bar for 24-48 hr at 22 °C. Aliquots (10 µl) were taken at 6-12 hr intervals for observation by AFM or EM.

Dot blot assay: For soluble Aß, the lyophilized peptide was dissolved in HFIP, diluted in water 0.5 mg/ml, and used immediately before each experiment. Fibrils and soluble oligomers were prepared as describe above. 2 µl of each sample applied to a

nitrocellulose membrane, blocked with 10% non-fat milk in Tris-buffered saline (TBS) containing 0.01% Tween 20 (TBS-T), at room temperature for 1 h (or overnight at 4 °C), washed three times for 5 min each with TBS-T and incubated for 1hr at room temperature with the affinity-purified anti-oligomer antibody (0.1 ug/ml in 3% BSA in TBS-T) or serum (diluted 1:1,000 in 3% BSA TBS-T). The concentration of Tween 20 is 10-fold lower than is normally used, because higher concentrations of detergent interfere with the detection of soluble oligomers by anti-oligomer (data not shown). The membranes were washed three times for 5 min each with TBS-T, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Promega) diluted 1:10,000 in 3% BSA/ TBS-T and incubated for 1 hour at room temperature. The blots were washed three times with TBS-T and developed with ECL chemiluminescence kit from Amersham-Pharmacia (Piscataway, NJ). The same membrane was stripped by incubating it for 45 min at 65 °C using stripping buffer (100 mM 2-mercaptoethanol, 2%SDS, 62.5mM Tris-HCl, pH6.7), washed 5 times for 5 min with TBS-T, blocked with 10% non-fat milk and immunodetected with 6E10 as described above. SH-SY5Y human neuroblastoma cells, were scraped in PBS, sonicated, the total protein concentration was diluted in PBS to 1.40 μg /μl. The cell lysate was divided into two tubes and protease inhibitor cocktail (Sigma P-2714) diluted 1:100 was added to one tube. For dot blots each dot contained 2.8 μg of total cell proteins plus the amount of Aβ oligomer or soluble indicated in the figure. The mixtures were spotted immediately after mixing Aß with the lysate.

ELISA assay: Samples were diluted to the desired concentration in coating buffer (0.1 M sodium bicarbonate, pH 9.6), 100μl of the samples were added to wells of 96-well microplates, incubated for 2 hours at 37 °C, washed three times with (PBS containing

0.01% Tween 20, PBS-T) and then blocked for 2 hr at 37 °C with 3% BSA TBS-T. The BSA used was IgG free (Sigma). The plates were then washed three times with PBS-T and 100 µl of anti-oligomer antiserum (1:10,000 dilution in 3% BSA/ TBS-T) was added and incubated for 1 hour at 37 °C. The plates were washed three times with PBS-T and 100 µl horseradish peroxidase-conjugated anti-rabbit IgG (Promega diluted 1:10,000 in 3% BSA TBS-T) was added and incubated for 1 hour at 37 °C. the plates were washed three times with PBS-T and developed using 3,3′,5,5′-tetramethylbenzidine (TMB, KPL Gaitherburg, MD). The reaction was stopped with 100µL 1M HCl and the plates were read at 450 nm. For ELISA measurements on cell SH-SY5Y lysates, each well contained 100µl (140µg) of total cell lysate proteins plus the amount of Aβ oligomer or soluble indicated in the figure.

Toxicity assays: SH-SY5Y human neuroblastoma cells were maintained in DMEM with 10mM HEPES, 10% fetal bovine serum, 4mM glutamine, penicillin (200unit/ml) and streptomycin (200µg/ml) in 5% CO_2 at 37° C. The medium was replaced every 2 days. Cells were differentiated in serum-free DMEM medium with N2 supplement and $1x10^{-5}$ M all-trans retinoic acid before use. Cells were plated at (10,000 cells/well) in 96-well plates and grown overnight. The medium was removed and Aß fibrils, soluble oligomers and soluble low MW Aß were added in 80 µl new medium without phenol red. After incubation for 4 h at 37 °C, the cells were assayed using an MTT toxicology kit (Tox-1) (Sigma) according to the manufacturer's directions. For LDH toxicity assays, SH-SY5Y cells were treated as described above. After 8 h at 37 °C, the LDH assay was performed using the LDH toxicology assay kit (Tox-7) (Sigma) according to the manufacturer's directions. Each data point was determined in triplicate and the standard deviation did not exceed 5%. For the inhibition of soluble oligomer toxicity by anti-oligomer antibody, samples were preincubated with an 8-fold molar excess of affinity purified anti-oligomer

antibody, polyclonal rabbit anti-α-synuclein antibody (Cat# AB5038), from Chemicon (Temecula, CA), mouse monoclonal anti-amylin (IAPP) antibody (Cat# RDI-amylin), from Research Diagnostics, inc. (Flanders, NJ) and affinity purified total rabbit IgG (from non-immunized rabbits),

Atomic force microscopy (AFM): Aliquots of protein stock solutions were prepared as described above and deposited onto freshly cleaved mica (Polysciences, Inc., Warrington, PA). Specimens were thoroughly examined using a TM Microscopes/Veeco AutoProbe® CP Research scanning probe microscope operating in the noncontact mode. A piezoelectric scanner with a range up to 100 μm was used for all images. The scanner was calibrated in the xy directions using a 1.0 μm grating, and in the z direction using several conventional height standards (5). The tips used were V-shaped silicon 2 μm cantilevers (Ultralevers, Model No. APUL-20-AU-25, Park Scientific Instruments) with a force constant of 2.1 N/m and resonant frequency ω_0 of approximately 80 kHz.

Immunofluorescence and Confocal Microscopy: Autopsy brain tissue from 7 moderate to severe AD cases (age at death ranging from 74 to 92 years) and 3 samples from non-demented elderly controls (age at death ranging from 74 to 95 years) was used in this study. The brain tissue used in this project was provided by the Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine. Brains were immersion fixed in 10% buffered formalin for 48-72 hours and the hippocampus with entorhinal cortex was dissected and stored in phosphate buffered saline (PBS) with .05% sodium azide at 4 °C until used. Blocks were stored in phosphate buffered saline (PBS) with .05% sodium azide at 4 °C. Free-floating 50 um thick serial sections were subsequently collected using a vibratome. Serial free-floating sections were pretreated with 90% formic acid for 4 minutes and then with 3% hydrogen peroxide in 10%

methanol to block endogenous peroxidase activity. Pilot studies indicated that formic acid pretreatment had little effect on anti-oligomer immunostaining but significantly improved anti-Aβ1-16 (6E10) immunostaining. Sections were subsequently incubated in biotinylated affinity purified anti-oligomer antibody (1:200) overnight at room temperature. For confocal microscopy, biotinylated anti-oligomer antibody was visualised using streptavidin conjugated Cy-3 fluorochrome (Jackson ImmunoResearch Labs, 1:200). One section was subsequently incubated in anti-Aβ1-16 (6E10; Signet Laboratories, Inc., Dedham, MA; 1:2000) and visualized with goat anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR; 1:200). A second section was counterstained with 0.5 % thioflavin-S in 50% ethanol after anti-oligomer immunostaining. Sections were coverslipped using Vectashield mounting media (Vector Laboratories Inc, Burlingame, CA). Control sections with either the primary or secondary antibodies omitted were negative.

Confocal images were collected on an Olympus IX70 inverted microscope using a 10 x, 20 x and 40 x objective for image analysis. Barrier filters at 510 and 605 nm were used. Channel 1 was used to acquire thioflavin-S or 6E10 fluorescence and Channel 2 was used to collect Cy-3 immunofluorescence. Each channel was collected separately and files were merged at the end of the experiment. A z-series scan at 2 μm intervals from individual plaques was captured to determine the spatial co-localization characteristics of anti-oligomer and either thioflavin-S or 6E10 positive plaques.

Dot blot analysis of human brain lysates: Frontal cortex tissue from 5 normal human controls, 5 AD patients and 2 individuals diagnosed with "Mild Braak Changes" (MBC), was homogenized in PBS containing a cocktail of protease inhibitors (diluted 1:100,

Sigma P-2714) and ultracentrifuged at 78,400 x G for 1 hour at 4 °C. The brain tissue used in this project was provided by the Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine. The supernatant was collected and stored at 4 °C. Total protein concentration was determined by BCA assay and the concentration of the samples was normalized. Two µl of sample containing 0.75 µg protein were then spotted onto the Protran nitrocellulose membrane and allowed to dry at room temperature. An additional 2 µl of sample were spotted onto the membrane and allowed to dry. The membrane was then placed into 10% non-fat milk-TBS-T solution for 1 hour at room temperature. The blot was washed 3 times for 5 minutes each and then placed into the anti-micellar Aβ antibody (1:10,000) in 3% BSA-TBS-T solution overnight at 4 °C. The membrane was washed and placed in anti-rabbit-HRP conjugated antibody solution (1:12,000) for 1 hour at room temperature. The membrane was again washed and exposed to ECL solution for 1 minute. After proper placement of the membrane in the film cassette, the film was exposed to the membrane for 30 seconds and the film was developed.

Supporting Text

Antigen preparation: Our previous studies indicated that oligomer formation coincides with the formation of a hydrophobic environment into which hydrophobic fluorescent dyes partition (6) and that the carboxyl terminus of soluble oligomeric Aß is much more shielded from the aqueous environment than it is in the fibrillar state, suggesting that it is buried in the interior of the micelle (7). We mimicked this structural organization of Aß in micellar oligomers by attaching the C-terminus of Aß40 to colloidal gold particles via a thioester bond (Fig. S1A). The resulting molecular mimics (Fig. S1B) are of the same approximate size as the naturally formed oligomeric intermediates (Fig. S1C) and have the same ß-sheet secondary structure and properties as determined by circular dichroism, thioflavin T fluorescence and ANS binding (data not shown). They are very stable, homogeneous and without contamination from either low MW Aß or fibrils (Fig S1B).

Antibody specificity: The specificity of the anti-oligomer antibody was also examined by screening lysates of SH-SY5Y cells for any cellular proteins that might react with the antibody by dot blot and ELISA assays. No detectable reactivity was observed for 2.8 ug of total cell protein on dot blots (Fig. S2a) or with 20 ug of total cell protein by ELISA assay (Fig. S2b), although approximately 0.75-6 ng of soluble Aß oligomers is readily detected when mixed with total cell lysate. The detection of the added soluble oligomers when mixed with cell lysate depends on the presence of protease inhibitors, suggesting that soluble oligomers are sensitive to proteolysis as has been previously reported (8). The unfractionated serum produced in response to repeated immunization with the molecular mimic is remarkably specific for the pathological micellar conformations of the amyloid forming peptides. This suggests that it may provide a means for vaccine development that avoids undesirable inflammatory side effects that have been observed for vaccination using Aß, since it specifically targets the

toxic micellar intermediates without any reactivity against APP or the monomeric Aβ or fibrillar deposits (9). The finding that soluble oligomers of all amyloids tested are all recognized by this antibody suggests that a vaccine directed against this epitope may be an effective therapeutic approach for a broad spectrum of amyloid diseases.

Rescue of cell toxicity by anti-oligomer antibody. We also examined the inhibition of soluble oligomer mediated SH-SY5Y cytotoxicity by anti-oligomer antibody using LDH release as a measure of cell lysis. Soluble oligomers of all types tested, but not low MW protein or fibrils, exhibited significant cytotoxic activity by LDH release (Fig. S3a). The toxicity was reversed by preincubation with anti-oligomer antibody, but not an equivalent amount of non-immune IgG. Fabs derived from anti-oligomer antibody were equally effective in inhibiting cell toxicity (Fig. S3b). It is interesting that soluble oligomers from normally cytosolic proteins, like α-synuclein and polyglutamine-containing proteins are toxic to cells when they are applied to the outside. Stefani and coworkers also reported that soluble oligomers derived from the SH3 domain of cytosolic PI3 kinase are also cytotoxic when applied externally (10). Although this situation is artificial, the fact that the oligomers are still toxic when applied on the inappropriate side of the membrane is consistent with the idea that the normally cytosolic and extracellular oligomers share a common mechanism of toxicity that is independent of their compartmentalization.

Identification of soluble oligomers in human AD brain. Human AD brain and control brain tissue was examined for the presence of soluble oligomers using confocal immunofluorescence and dot blots of soluble brain homogenates. We examined soluble lysates of human of brain tissue (5 non-AD controls, 2 individuals with MBC, and 5 individuals with AD for the presence of soluble oligomers. No anti-oligomer

immunoreactivity is observed for the 5 non-demented controls (Fig. S4), but the 2 MBC samples and the 5 AD samples all contain detectable amounts of soluble oligomers. The amount of immunoreactivity observed is variable. In a larger sample of 24 AD patients, 17 of the samples contained detectable amounts of soluble oligomers (data not shown). The explanation for this variation in the amount of soluble oligomers is not simply due to variation in post-mortem interval and is currently under investigation. Using confocal immunofluorescence, anti-oligomer immunofluorescence is occasionally observed in the immediate vicinity of large diffuse plaques identified by 6E10 immunofluorescence. The online video shows a z-series through a diffuse plaque labeled first with anti-oligomer (red) followed by 6E10 (green) (Video S5). The oligomer fluorescence is localized at the periphery of the diffuse plaque and does not overlap with the 6E10 fluorescence. This is consistent with the non-overlapping distributions of anti-oligomer and thioflavin-S fluorescence shown in Fig. 4. This lack of co-localization of oligomers and thioflavin S is not inconsistent with the report by Stefani and coworkers that soluble oligomers are thioflavin T positive because the fluorescence emission of thioflavin S is not altered upon fibril association (3) and it is not yet clear whether oligomers bind thioflavin S.

Supporting Figures

Fig. S1.

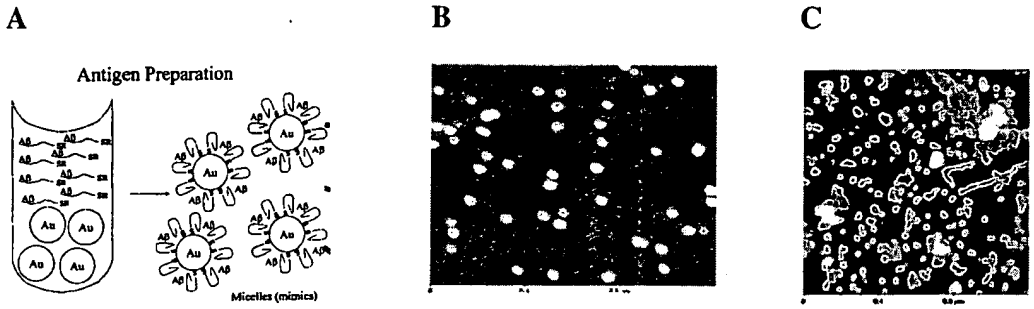

Fig. S1. Antigen preparation and characterization. **A**. Schematic representation of the antigen preparation. **B**. AFM image of the micelle molecular mimic used as an antigen. **C**. AFM image of the soluble oligomers prepared from Aβ 40. The curvilinear structures that appear to represent strings of the spherical oligomers are commonly referred to as "protofibrils".

Fig. S2